



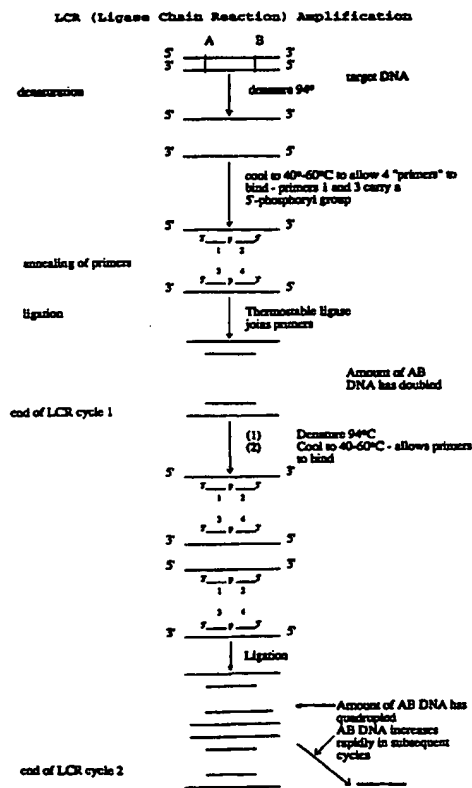
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> :  C12Q 1/68	A1	(11) International Publication Number: <b>WO 93/09250</b>  (43) International Publication Date: 13 May 1993 (13.05.93)
<p>(21) International Application Number: PCT/AU92/00587</p> <p>(22) International Filing Date: 30 October 1992 (30.10.92)</p> <p>(30) Priority data: PK 9224 1 November 1991 (01.11.91) AU</p> <p>(71) Applicants (for all designated States except US): ADELAIDE CHILDREN'S HOSPITAL [AU/AU]; UNIVERSITY OF SOUTH AUSTRALIA [AU/AU]; 72 King William Road, North Adelaide, S.A. 5006 (AU).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): MORRIS, Charles, Phillip [AU/AU]; 72 King William Road, North Adelaide, S.A. 5006 (AU). HARRIS, Raymond, John [AU/AU]; University of South Australia, North Terrace, Adelaide, S.A. 5000 (AU).</p>		<p>(74) Agent: R.K. MADDERN &amp; ASSOCIATES; 345 King William Street, Adelaide, S.A. 5000 (AU).</p> <p>(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).</p> <p><b>Published</b> With international search report With amended claims</p>

## (54) Title: SOLID PHASE AMPLIFICATION PROCESS

## (57) Abstract

An assay and method of use thereof for amplification and detection of target nucleic acids in patient samples, which is rapid, simple and accurate is described. The assay and method utilizes any one of a variety of amplification methods, in combination with primers immobilized onto either the container containing the patient sample, such as a microtiter well plate, or a dipstick which is readily immersible into the container. Amplification occurs in the presence of both the first, immobilized primer and a second, labelled primer which hybridizes to the target nucleic acid in the opposite direction to the first. Examples demonstrate the effectiveness of the method in detecting a specific species of mycoplasma, as well as the gene characteristic of cystic fibrosis.



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LJ	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

## SOLID PHASE AMPLIFICATION PROCESS

### BACKGROUND OF THE INVENTION

The present invention is generally in the field of methods for the detection of nucleic acid sequences through amplification and hybridization to labelled probes.

Hybridization methods are widely utilized in testing for the presence of particular nucleic acid sequences, identifying and/or quantifying such sequences. Hybridization occurs when there is sufficient complementarity of two nucleic acid sequences to effect non-covalent binding between the two molecules.

Various improvements and modifications have been introduced to increase the specificity and sensitivity of the reaction. In particular, polymerase chain reaction (PCR) techniques have been used to amplify the target nucleic acid sequence. In a recent development, two consecutive sets of PCR cycles ("nested PCR") have been used to both amplify and label the target nucleic acid sequence. The labelled sequence is then immobilized on a solid phase carrier, and testing is carried out using a reagent specific to the label. (See DJ Kemp, et al., "Colorimetric detection of specific DNA segments amplified by polymerase chain reactions", Proc. Natl. Acad. Sci. USA 86, pp. 2423-2427, 1989).

WO89/11546 by Paulsen, et al., describes a method using PCR with DNA primers, one of which is soluble and the other which is bound to superparamagnetic particles, allowing greater ease of separation of the amplified product which facilitates detection. While the nucleic acid is bound to the particle, however, amplification via the beads is still taking place in a soluble phase, ie the beads are suspended in a liquid phase, increasing the time and complexity of the reaction, and requiring equipment for isolation of the superparamagnetic particles.

AU A-47144/89 by Molecular Diagnostics, Inc. also reports a method for increasing detection of nucleic acids using PCR to amplify sequences, where one or both of the primers are in solution or immobilized or immobilizable, for example, to Sephadex, to facilitate detection. The process can be used to yield immobilized target nucleic acid.

However, prior art methods for amplifying nucleic acid sequences have all suffered from problems associated with carrying out reactions in the liquid, rather than solid, phase. After each step of the reaction, the intermediate product must be isolated, before being reacted in the next step. Isolation steps are cumbersome, time consuming and often involve labile linkage reagents. In addition, no isolation step can be completely successful.

It is therefore an object of the present invention to provide a method which is rapid, simple, and provides an accurate, sensitive means for detecting small amounts of nucleic acid in a test sample.

It is a further object of the present invention to provide an assay which is relatively inexpensive, simple, rapid, and provides an accurate, sensitive means for detecting small amounts of nucleic acid in a test sample in clinical and research laboratories.

#### SUMMARY OF THE INVENTION

The present invention utilizes the concept of amplification of a target nucleic acid by means of two primers, one of which is directly immobilized onto a sample container surface, and the other which is labelled for ease of detection. By definition, both primers must be hybridizable with a target nucleic acid sequence. According to the method for determining the presence of a target nucleic acid sequence in a sample, the sample is placed in a container with the immobilized nucleic acid

primer. The target sequence is then amplified using a second labelled primer or labelled polymerase substrate which, on incorporation, allows detection and quantification via the label on the solid phase amplified nucleic acid sequence.

Examples demonstrate the detection of the gene for cystic fibrosis and a specific type of mycoplasma in patient samples.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a schematic of the PCR (Polymerase Chain Reaction).

Figure 2 is a schematic of the LCR (Ligase Chain Reaction).

Figure 3 is a schematic of the NASBA (Nucleic Acid Sequence Based Amplification). Thin lines, RNA; thick lines, DNA; RT, reverse transcriptase.

Figure 4 is a schematic of the SPA method for amplification.

Figure 5a is a schematic of the target generation scheme for SDA.

Figure 5b is a schematic of automatically entering the SDA reaction cycle.

Figure 6 is a schematic of the reaction products of the modified SDA process.

#### **DETAILED DESCRIPTION OF THE INVENTION**

The assay and method of use described herein have the following elements: a target molecule to be detected in any test or patient sample, a first primer which is immobilized to a solid surface suitable for containment of the patient sample and is hybridizable to the target molecule, a second primer which can be detected using a variety of different methodologies and is hybridizable to the target molecule, and means for amplification of the target sequence via the two primers.

A common problem in laboratories utilizing diagnostic methods based on nucleic acid amplification is the occurrence of

**SUBSTITUTE SHEET**

false positives. This problem invariably arises due to the contamination of the laboratory with amplified target molecules (amplicons). Of relevance here is the intrinsic high level of amplicon containment offered by solid phase amplification (SPA). SPA requires very little manipulation of solution phase amplicons. Such amplicons are removed from SPA reactions by simple washes and can be readily destroyed. Additionally, the solid phase amplicons offer little or no contamination threat. This level of containment is unmatched by any other amplification process. Thus SPA offers a high degree of amplicon containment.

#### The Target Molecule

This method allows detection and identification of virtually any nucleic acid sequence, and thus allows the detection and identification of viruses, microorganisms and parasites, the detection of genetic diseases, either by detection of sequence variations (mutations) which cause or are associated with a disease or are linked (Restriction Fragment Length Polymorphisms or RFLP's) to the disease locus, and sequence variations which are associated with, or cause, cancer, and the detection and identification of nucleic acid sequences for forensic fingerprinting, tissue typing and for taxonomic purposes, namely the identification and speciation of microorganisms, flora and fauna, and for the production of solid phase nucleic acid templates for DNA and RNA sequencing. The methodology is also useful for other applications.

The following are a few examples of such applications :

1. Pathogen Detection

Detection of viruses (HIV, hepatitis viruses, papilloma).

Detection of microorganisms (Mycobacteria, Legionella, Mycoplasmas).

(Applications in clinical medicine, veterinary science, aquaculture, horticulture and agriculture).

2. (a) Detection of sequence variations which cause or are associated with a disease or are genetically linked to a disease locus. Examples cover genetic diseases and cancer.  
e.g. Point mutations - sickle cell anaemia, phenylketonuria, Lesch Nyhan syndrome.  
Small deletions - Cystic fibrosis (F508).  
Large deletions - thalassaemias, Duchenne muscular dystrophy.  
Sequence reiteration - Fragile X.  
RFLP's (Restriction Fragment Length Polymorphisms) - Huntington's chorea, thalassaemias, cystic fibrosis.  
(b) Population screening for carriers of serious genetic diseases, where the incidence of carriers is high and the number of mutations is low, e.g., cystic fibrosis, and Tay Sach's disease in Ashkenazi Jews.
3. Cancer - detection of predisposing sequences, e.g., Philadelphia chromosomes in chronic myelogenous leukemia, antibody gene rearrangements, specific deletions; detection and monitoring of treatment, remission and relapse.
4. Tissue typing - determination of HLA genotypes.
5. Forensic applications - e.g., VNTR DNA fingerprinting of individuals and obtaining DNA fingerprints from forensic samples.
6. Maternity and paternity testing.
7. Foetal sex determination
8. Taxonomy - Classification and speciation of organisms.

9. Quality control in agriculture and the food and pharmaceutical industries, e.g., assessment of authenticity of food products.

The assay and method of use described herein are equally applicable to RNA (ribonucleic acid) sequences, as well as DNA (deoxyribonucleic acid) sequences, although it may be necessary to produce a cDNA copy of RNA sequences.

#### Primers

The primers are synthesized to be complementary to the target molecule. In general these are approximately 15 to 30 nucleotides in length and flank a portion of the target molecule. One hybridizes to the 5' end of a portion of the target molecule, the other to the 3' end of a portion of the target molecule. All appropriate reagents and equipment are commercially available.

#### Solid Phase Support

One primer is attached using known methodology, as described below, to a solid phase support. The advantages of the system described herein is that the solid support forms or is readily insertable into the container for the patient sample, eliminating the need for separate centrifugation, magnetic collection, filtration or other separation steps, as required by the prior art methods, for example, to separate superparamagnetic beads. A preferred material is the microtiter well plates that are commercially available.

The solid support may be any structure having a surface which can be derivatised with a nucleic acid primer, such that the primer can participate in solid phase nucleic acid amplification. In the preferred embodiment, the solid support is a planar solid phase material, for example, the side of a microtiter well or a dip stick.

Specific preferred supports include :

1. The inside surface of wells of any microtitre trays, e.g., Covalink trays - Nunc.



2. Microtitre tray lids with protrusions which fit into microtitre trays, e.g. Fast Elisa dish - Falcon.
3. Dipsticks for low volume applications, e.g., suburban and country medical practices.

These all offer the potential for high volume diagnostic applications for solid phase amplification (SPA), especially if isothermal (single temperature) amplification is used.

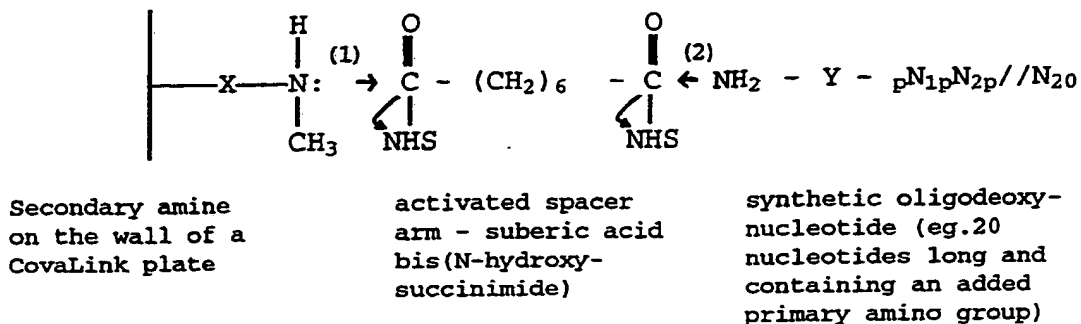
It is surprising that solid phase amplification of nucleic acids on the surface of a microtiter well produces a significant amount of amplified product due to the inherently less favourable kinetics of solid phase molecular interactions, versus those of the solution phase. The results demonstrate that, despite the slightly slower rate of amplification using a solid phase as compared with solution phase, or with bead-based amplification, which offers a vary large surface area, the system offers a rapid, sensitive, and clinically viable method of nucleic acid amplification.

Although described herein as preferentially involving binding of the first primer to the solid phase before initiation of amplification, in some cases it may be desirable to combine conventional liquid phase amplification techniques with the solid phase amplification (SPA) procedures. For instance, if the target nucleic acid molecule is only likely to be present in very small quantities, then it maybe beneficial to carry out an initial liquid phase amplification in the vessel to which the primer is bonded. This liquid phase amplification step may involve a small proportion of the first primer or it may involve other primers. When the target molecule is amplified to a sufficient level, then the SPA process takes over.

#### Anchoring of the First Primer to Solid Phase Support.

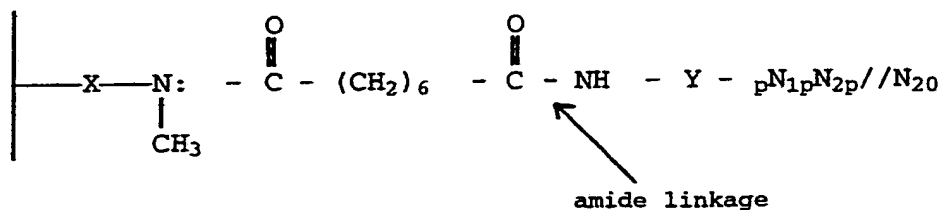
Any method for linking nucleic acid sequences to solid supports may be used. For example, any of the following methods are suitable :

1. Amide linkage of primer to a solid phase



Derivatisation sequence :

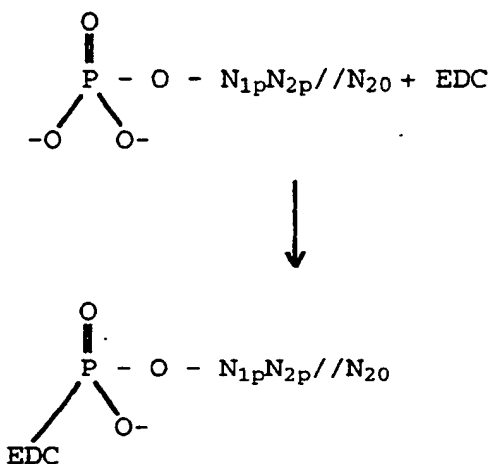
- (a) The surface bound (solid phase) secondary amine (on walls of wells of CovaLink plates) is reacted with the activated spacer arm.
- (b) The solid phase activated spacer arm is reacted with the primary amino group of the synthetic oligodeoxynucleotide, to form the product, which is a solid phase primer (amide linkage), as shown below.



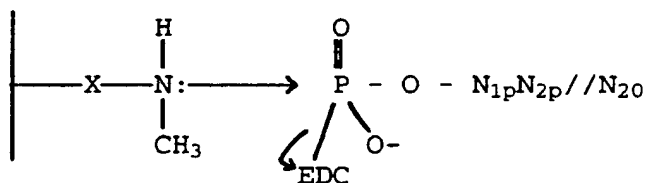
# SUBSTITUTE SHEET

2. Amidate linkage of primer to a solid phase

- (a) A 5'-phosphorylated oligodeoxynucleotide (e.g. 20 nucleotides long) is reacted with a phosphate activating compound, preferably a water-soluble carbodiimide such as EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide).

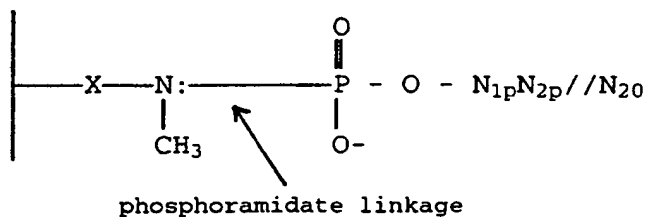


Intermediate (activated oligodeoxynucleotide)



Secondary amine in  
CovaLink well

Activated  
oligodeoxynucleotide



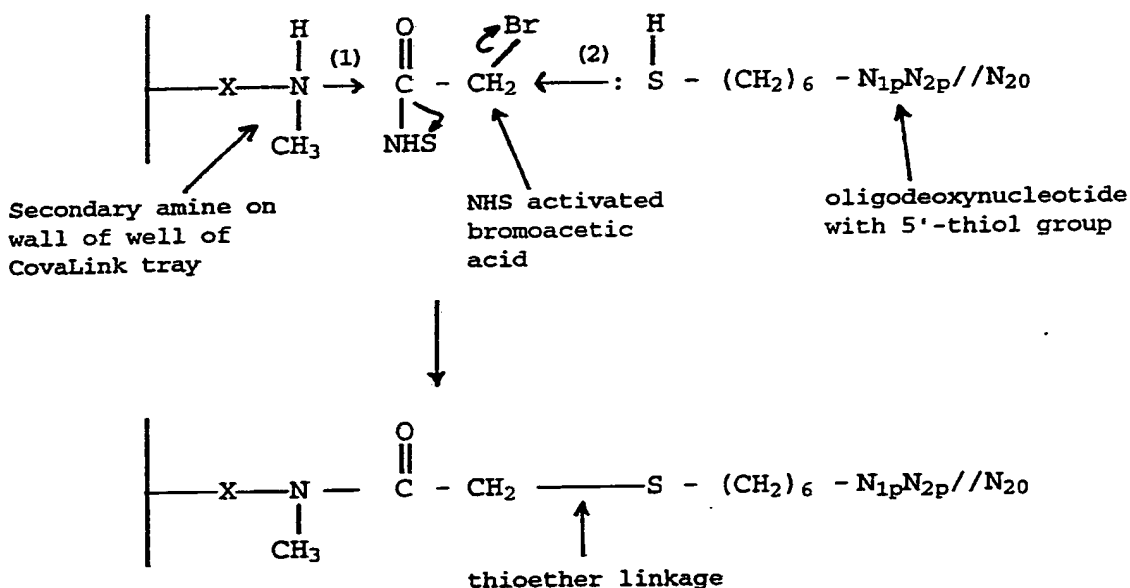
Product - solid phase primer (phosphoramidate linkage)

**SUBSTITUTE SHEET**

### 3. Thioether linkage of primer to a solid phase

The reaction sequence is as follows :

- (a) Bromoacetic acid - NHS is reacted with the secondary amino group on the solid support.
- (b) The thiol group of the oligodeoxynucleotide is reacted with the bromomethyl group of the intermediate product formed in step (a).



Product solid phase primer (thioether linkage).

### 4. Introduction of amino groups onto the surface of microtitre tray wells

Plates were nitrated with acetyl nitrate using the method of R. Taylor, "Electrophilic aromatic substitution" p. 269 (J. Wiley and Sons 1990). Reduction was via  $\text{SnCl}_2$ .

#### Amplification Procedures

Currently known methods of nucleic acid amplification include :

- (a) PCR (Polymerase Chain Reaction).
- (b) LCR (Ligation Chain Reaction).
- (c) NASBA (Nucleic Acid Sequence Based Amplification), using RT/RNase H/T7 polymerase.

RT = reverse transcriptase.

RNAse H = ribonuclease H

T7 polymerase = T7 bacteriophage RNA polymerase.

(d) Q $\beta$  replicase based amplification.

(e) Strand displacement method (SDA).

The principles of some of these amplification methods are described below.

#### PCR (Polymerase Chain Reaction)

Polymerase chain reaction procedures and reagents are commercially available and described in the literature, for example, by R.K. Saiki, et al., "Enzymatic Amplification of  $\beta$ -globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia", Science 230, pp. 1350-1354. Refer to Figure 1.

#### LCR (Ligation Chain Reaction)

The ligation chain reaction is well known and described in the literature, for example, by U. Landegren, et al., "A Ligase-mediated Gene Detection Technique", Science 241, pp. 1077-1080, 1988. Refer to Figure 2.

NASBA (Nucleic Acid Sequence Based Amplification)

This method is also well established in the literature, as described, for example, by J. Cuatelli, et al., "Isothermal in Vitro Amplification of Nucleic Acids by a Multienzyme Reaction Modelled After Retroviral Replication", Proc. Natl. Acad. Sci. USA 87, pp. 1874-1878, 1990.

The strategy of the NASBA scheme is shown in Figure 3. The reaction depends on a continuous cycle of reverse transcription and transcription reactions to replicate an RNA target by means of cDNA intermediates. DNA targets can also be amplified. Primers 1 and 2 prime DNA synthesis and Primer 1 encodes the promoter sequence for the T7 RNA polymerase (black boxes). Steps 1-6 depict the synthesis of a double-stranded cDNA, which is a transcription template for T7 RNA polymerase. Complete cDNA synthesis is dependent on the digestion of the RNA in the intermediate RNA, DNA hybrid (step 4) by RNase H. Transcription-competent cDNAs yield antisense RNA copies of the original target (step 7). These transcripts are converted to cDNAs containing double-stranded promoter on one end (steps 7-12).

These cDNAs yield antisense RNAs, which can re-enter the cycle.

## CHARACTERISTICS OF NUCLEIC AMPLIFICATION SYSTEMS

	One primer incorp- orated	Two or more primers incorp- orated	Double- stranded product	Can be labelled at non- anchored end	Can be internally labelled	Requires temperature cycling
1. PCR	+	+	+	+	+	+
2. LCR (Ligase chain reaction)	+	+	+	+	+	+
3. NASBA* RT/T7	+	+	+	+	+	No 42° only**
4. QB						No**
5. SDA	+	+	+	+	+	No**

\* NASBA = Nucleic acid sequence based amplification.

\*\* Big technical advantage.

The process of solid phase amplification (SPA) of a target nucleic acid sequence, using PCR as the method for amplification, is illustrated in Figure 4.

One primer is immobilized to the wall of the plate, and the other is labelled with a fluorescent label. As shown by this diagram, generally 20-30 cycles are needed to amplify sufficient DNA for detection. The plate is then washed to remove unincorporated primer 2 and the plate read using a plate fluorimeter. A positive result indicates the presence of the target nucleic acid, a negative result indicates that the target nucleic acid is not present.

**SUBSTITUTE SHEET**

SDA (Strand Displacement Amplification)

The following scheme is taken directly from G.T. Walker, et al., "Strand displacement amplification - an isothermal, in vitro DNA amplification technique", Nuc. Acid Res. 20, pp. 1691-1696, 1992.

The target generation scheme for SDA is shown in Figure 5a. This figure depicts the initial steps in an SDA reaction which transform the original target sequence into the amplification cycle depicted in Figure 5b. A target DNA is heat denatured. Four primers (B<sub>1</sub>, B<sub>2</sub>, S<sub>1</sub> and S<sub>2</sub>), present in excess, bind the target strands at positions flanking the sequence to be amplified. Primers S<sub>1</sub> and S<sub>2</sub> have *HincII* recognition sequences (5' GTTGAC) located 5' to the target complementary sequences. The four primers are simultaneously extended by exo-klenow using dGTP, dCTP, TTP and dATPS. Extension of B<sub>1</sub> displaces the S<sub>1</sub> primer extension products, S<sub>1</sub>-ext. Likewise, extension of B<sub>2</sub> displaces S<sub>2</sub>-ext. B<sub>2</sub> and S<sub>2</sub> bind to displaced S<sub>1</sub>-ext. B<sub>1</sub> and S<sub>1</sub> bind to displaced S<sub>2</sub>-ext. Extension and displacement reactions on templates S<sub>1</sub>-ext and S<sub>2</sub>-ext produce two fragments with a hemiphosphorothioate *HincII* at each end and two longer fragments with a hemiphosphorothioate *HincII* site at just one end. *HincII* nicking and exo-klenow extension/displacement reactions initiate at these four fragments, automatically entering the SDA reaction cycle depicted in Figure 5b. Sense and antisense DNA strands are differentiated by thin and thick lines. *HincII* recognition sequences are depicted by (thin line- thick line-thin line).

The SDA reaction cycle is shown in Figure 3b. These reaction steps continuously cycle during the course of amplification. Present in excess are two SDA primers (S<sub>1</sub> and S<sub>2</sub>). The 3'-end of S<sub>1</sub> binds to the 3'-end of the displaced target strand T<sub>1</sub>, forming a duplex with 5' - overhangs. Likewise, S<sub>2</sub> binds T<sub>2</sub>. The 5'-overhangs of S<sub>1</sub> and S<sub>2</sub> contain the *HincII* recognition sequence (5' GTTGAC). Exo-klenow extends the 3'- ends of the duplexes using dGTP, dCTP, TTP and dATPS, which produces hemiphosphorothioate recognition sites on S<sub>1</sub>.T<sub>1</sub> and S<sub>2</sub>.T<sub>2</sub>. *HincII* nicks the unmodified primer strands of the hemiphosphorothioate recognition sites, leaving intact the modified complementary strands. Exo-klenow



extends the 3'-end at the nick on S<sub>1</sub>.T<sub>1</sub> and displaces the downstream strand that is equivalent to T<sub>2</sub>. Likewise, extension at the nick on S<sub>2</sub>.T<sub>2</sub> results in displacement of T<sub>1</sub>. Nicking and polymerization/displacement steps cycle continuously on S<sub>1</sub>.T<sub>1</sub> and S<sub>2</sub>.T<sub>2</sub> because extension at a nick regenerates a nickable *HincII* recognition site. Target amplification is exponential because strands displaced from S<sub>2</sub>.T<sub>2</sub> serve as target for S<sub>1</sub>. Sense and antisense DNA strands are differentiated by thin and thick lines. Intact and nicked *HincII* recognition sequences are depicted by (thin line-thick line-thin line) and (thin line-thick line, space, thick line-thin line), respectively. The partial *HincII* recognition sequence 5' GAC and its complement 5' GTC are present at the 5'-and 3'- ends of displaced strands as represented by (thick line-thin line) and (thin line-thick line).

The following modifications of SDA (see Walker et al., 1992 Nuc. Acid Res 20, 1691-1696) is required to allow implementation of SPA.

These modifications should be read in conjunction with Figure 5.

- 1A Primers S<sub>1</sub> and S<sub>2</sub> are chemically prepared to contain *HincII* recognition sequences which are resistant to cutting by this enzyme, i.e., primers S<sub>1</sub> and S<sub>2</sub> are chemically prepared to contain phosphorothioate nucleotides using known methodology.
- 1B Primer S<sub>2</sub> carries a detector tag, introduced during chemical synthesis, for example, a 5'-biotin group or 5'-fluorophore.
- 2 Only standard dNTPs are then required for amplification (i.e., dATPS are not be used).

With regard to the SPA version of SDA the process proceeds as follows :

- (a) Primer S<sub>1</sub> is covalently bound via the 5'-end to the microtitre well surface.
- (b) Biotinylated primer S<sub>2</sub> is free in solution.
- (c) SPA-SDA isothermal amplification is followed by a simple wash to yield a final solid phase double stranded product as shown in Figure 6.

- (d) The label is then used to detect the bound target molecule. For example biotin would then be detected colorimetrically via avidin-horseradish peroxidase or fluorimetrically via avidin-Europium.

It is also possible to improve the amount of solid phase product produced by the inclusion of a small quantity of primer S1 in the solution phase to increase the quantity of the target in solution.

#### Detection Labels

Any suitable method for labelling nucleic acid sequences can be used, e.g., fluorescent labelling, biotin labelling or enzyme labelling. The label is introduced into the anchored amplified nucleic acid product either via labelled primer 2 or via labelled nucleotide substrates.

Exemplary detection systems include:

(a) Fluorescent labelling and detection

Source of fluorescent label :

- (i) Fluorescent primer 2, e.g., fluorescein-primer 2.
- (ii) Fluorescent derivatives of ribo or deoxyribo-NTPs (substrates for RNA and DNA polymerases, respectively).

SPA Product : fluorescently labelled anchored nucleic acid.

Detection system : fluorescent plate reader or other fluorescence detection system.

(b) Biotin label

- (i) Biotinylated primer 2.
  - (ii) Biotinylated dNTPs, e.g. biotinylated dATP.
- SPA product : Biotin labelled anchored nucleic acid.

Detection systems :

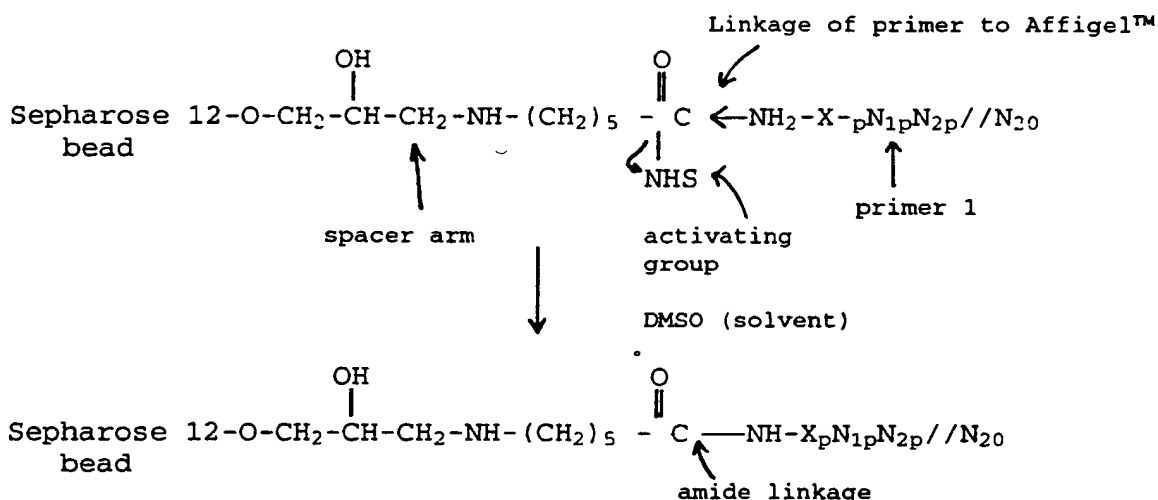
- (i) Europium labelled avidin, with quantitation of Europium by time resolved fluorescence spectroscopy.
- (ii) Avidin - horseradish peroxidase, with quantification of the resulting coloured product by spectroscopy (e.g., plate reader).

- (iii) Avidin - alkaline phosphatase, with quantitation of the resulting coloured or fluorescent product by standard or fluorescent spectroscopy.

Other labels include radiolabels, other enzyme systems, and dyes, as well as nucleotides with altered emission spectra.

The present invention is further demonstrated by reference to the following non-limiting examples.

**Example 1: Affigel anchored Solid Phase Amplification (SPA)**



**Product:**

Primer 1 bound to Affigel™ beads (amide linkage).

The DNA sequence to be amplified was a portion of the CFTR (cystic fibrosis transmembrane conductance regulator) gene.

Amplification was by the polymerase chain reaction (PCR).

The PCR conditions were as follows :

10 mM Tris-HCl (pH 8.3 at 25°C), 2.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1% v/v Triton x 100, 0.04% w/v gelatin, 0.4 mM dNTP's, Primer 2 250 ng, normal human DNA, 0.5µg (note : dried blood spot on paper diameter 3.0 mm was used for some reactions). Taq polymerase 2.5 units. Cycling conditions : 30 sec 94°C, 60 sec 72°C, 30 sec 55°C, 30 cycles.

In this particular embodiment, the SPA product was labelled with biotin, rather than a fluorescent label.

Detection of SPA Product

The Affigel<sup>TM</sup> bound product was detected by use of Europium labelled avidin and time resolved fluorescence spectroscopy. The results are shown in Table 1.

**Table 1: Results of CFTR gene Test.**

Affigel-primer 1 ( $\mu$ l)	Target	Test Counts	Denatured(a) Control Counts	Test counts Control Counts
1	0.5 $\mu$ g DNA	35,000	6,400	5.5
2	0.5 $\mu$ g DNA	74,000	3,800	20
5	0.5 $\mu$ g DNA	550,000	22,000	25
10	0.5 $\mu$ g DNA	1,300,000	110,000	12
20	0.5 $\mu$ g DNA	1,400,000	150,000	9
10	3 mm blood disc	60,000	6,000	10
20	3 mm blood disc	42,000	4,800	8.8
10	boiled extract of 3 mm blood disc	112,000	8,000	14
20	boiled extract of 3 mm blood disc	180,000	12,000	15
Control 10	No DNA	54,000	12,000	4.6
Control 20	No DNA	83,000	34,000	2.5

(a) The SPA product can be treated at pH 9.8 to dissociate the biotin labelled DNA strand from the primer anchored DNA strand.

Conclusion:

5-10 0.5  $\mu$ l of Affigel<sup>TM</sup>-primer 1 is an efficient solid support for SPA for either purified DNA or 3 mm blood discs.

**Example 2: SPA Detection of the CFA Phe 508 mutation**

The amplification reagents and conditions were as follows:

Primers:

Common primer carrying 5'-biotinyl group

5'-biotin-GTT GGC ATG CTT TGA TGA CGC TTC-3'.

Primer for detection of  $\Delta$  F 508 mutation

$\Delta$  F 508 5'-(T)<sub>10</sub> GGC ACC ATT AAA GAA AAT ATC ATT GG-3'.

Normal primer for detection of normal sequence

5'-phosphate-TTT TTT TTT GGA TCC GGC ACC ATT AAA GAA AAT  
ATC TT-3'.

Each SPA 50 µl reaction contained 67 mM Tris HCl pH 8.8 at 25°C, 16.6 mM ammonium sulphate, 0.45% Triton X-100, 200 µg/ml gelatin, 200 µM each of dATP, dCTP, dGTP, dTTP, 3.0 mM MgCl<sub>2</sub>, 250 ng of 5' biotin primer, 2.5 units Taq polymerase.

SPA was performed in Costar microtitre wells previously nitrated with acetic anhydride/nitric acid and reduced by tin chloride to give wells which carried amino groups covalently attached to the well walls.

The normal or ΔF 508 primers were covalently linked to amino groups on the well walls via the amide linkage method. Product detection was via avidin-Europium and time resolved fluorescence quantification and colorimetrically by avidin-horseradish peroxidase.

SPA cycle amplification conditions :

1 min 95°C  
1 min 57°C  
1 min 72°C

Initial denaturation for 7 minutes at 95°C and final extension for 10 minutes at 72°C.

**Table 2 : Results of SPA ΔF 508 Mutation screen.**

<u>Template</u>	<u>TRF Counts</u>	<u>Absorbance at 450 nm</u>
Wild type 500 bp PCR product	1,000,000	1.8
ΔF 508 500 bp PCR product	10,000	0.2
Wild type genomic DNA	45,000	0.8
ΔF 508 genomic DNA	10,000	0.15
Salmon sperm DNA (sonicated)	10,000	0.15
No template	10,000	0.2

Table 2 shows signals obtained with the wild type primer immobilized on the wells, following solid phase amplification of different templates. Additionally the signal with genomic DNA was increased to approximately 400,000 counts when 45 cycles were used. Additionally, when some of the solid phase primer was added in solution the signal was greatly increased from genomic as target.

For example, for detection of the normal Cystic Fibrosis gene, addition of normal primer to the solution gave the following results :

2 ng	normal primer in solution gave a product signal of	80,000 counts
5 ng	normal primer in solution gave a product signal of	1,000,000 counts
10 ng	normal primer in solution gave a product signal of	1,200,000 counts
20 ng	normal primer in solution gave a product signal of	1,200,000 counts

When the mutant  $\Delta F$  508 primer was immobilized signals obtained were similar to those in the table when  $\Delta F$  508 was used as a template.

**Example 3 :      Detection of mycoplasma pneumoniae**

- the major causative agent of atypical pneumonia.

**Primers**

Solution phase primer 5'-biotinyl - TC AAAA CAA CGA CAC- 3' corresponds to nucleotides 3863-3877 of the P1 gene.

Solid phase primer 5'-TTT CAG AAA GTC GAC-3' corresponds to nucleotides 4114-4100 of the P1 gene, 5'-phosphorylated with polynucleotide kinase and ATP.

**Preparation of DNA from M. Pneumoniae**

Various amounts (in colony forming units - CFU) of whole M. pneumoniae cells were added to normal nasopharyngeal aspirate and were collected by centrifugation and the pellet was treated with proteinase K

(30  $\mu$ l, 200  $\mu$ g/ml 10 mM Tris-HCl pH 8.3) at 37°C for 1 hour to liberate the DNA, then at 95°C to inactivate the proteinase K.

SDA reaction mix as for cystic fibrosis  $\Delta$ F 508 detection except primers were as given above.

#### Microtitre Plates

Plates derivatized with solid phase primer were prepared as for the cystic fibrosis  $\Delta$ F 508 detection system.

#### Amplification Conditions

30 cycles of amplification using :

90°C for 1 min  
40°C for 1 min  
60°C for 3 min

The initial denaturation was 7 min at 95°C and the final extension was 10 min at 72°C.

**Table 3 : Product detection was via Avidin-Europium and time resolved fluorescence quantification of Europium**

<b>M. Pneumoniae CFU</b>	<b>Counts</b>
10 <sup>7</sup>	593,000
10 <sup>6</sup>	263,000
10 <sup>5</sup>	280,000
-	48,000

The following organisms did not give a positive signal (signals 14,000 - 47,000) : M. buccale, M. fermentans, M. genitalium, M. hominis, M. hyorrrhinis, M. salivarium, A. laidlawii A, A. laidlawii B.

Additionally five patients with confirmed M. pneumoniae infection with greater than 10<sup>5</sup> CFU/ml of respiratory secretion gave positive signals 121,000-330,000 counts.

The results demonstrate the surprising superiority of the assay where the first primer is immobilized to a solid planar support (Examples 2 and 3), as compared with primer immobilized to beads (Example 1).

Modification and variations of the method and assay of the present invention will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the following claims.



We claim:

1. A method for detecting a target nucleic acid comprising:  
providing a first primer hybridizing to the target nucleic acid, wherein the primer is immobilized on a solid phase planar support, wherein the solid phase support forms a part of or is insertable into a container for the sample,  
providing a second primer hybridizing to the target nucleic acid in the opposite direction, wherein the primer is labelled with a detectable label,  
reacting the first and second primers with a sample containing nucleic acids under conditions which allow amplification of the nucleic acids that hybridize to the first and second primers in the container for the sample, and  
detecting the presence of bound second primer.
2. The method of claim 1 wherein the container is a microtiter plate well.
3. The method of claim 1 wherein the first primer is immobilized on a solid planar support which is immersible into the container.
4. The method of claim 1 wherein the nucleic acid is amplified by the polymerase chain reaction.
5. The method of claim 1 wherein the nucleic acid is amplified by the nucleic acid sequence based amplification.
6. The method of claim 1 wherein the nucleic acid is amplified by the Q $\beta$  replicase based amplification.
7. The method of claim 1 wherein the nucleic acid is amplified by the strand displacement amplification process.
8. The method of claim 1 wherein the nucleic acid is selected from the group consisting of nucleic acids specific to species of microorganisms, characteristic of a genetic disorder or disease, HLA types, and sex.
9. The method of claim 1 wherein the label is selected from the group consisting of fluorescent labels, biotin, radiolabels, enzymes specific for chromogenic substrates and dyes.

10. Any assay system for detecting a target nucleic acid comprising:
- a first primer hybridizing to the target nucleic acid, wherein the primer is immobilized on a solid phase planar support,
  - wherein the solid phase support forms a part of or is insertable into a container for the sample,
  - a second primer hybridizing to the target nucleic acid in the opposite direction,
  - wherein the primer is labelled with a detectable molecule,
  - reagents for amplification of the sample containing nucleic acids under conditions which allow amplification of the nucleic acids that hybridize to the first and second primers in the container for the sample.
11. The assay of claim 10 wherein the container is a microtiter well plate, further comprising the well plate.
12. The assay of claim 10 wherein the first primer is immobilized on a solid planar support which is immersible into the container.
13. The assay of claim 10 wherein the reagents for amplifying the nucleic acid are those for use in the polymerase chain reaction.
14. The assay of claim 10 wherein the reagents for amplifying the nucleic acid are those for use in the nucleic acid sequence based amplification.
15. The assay of claim 10 wherein the reagents for amplifying the nucleic acid are those for use in the Q $\beta$  replicase based amplification.
16. The assay of claim 10 wherein the reagents for amplifying the nucleic acid are those for use in the strand displacement amplification process.
17. The assay of claim 10 wherein the nucleic acid is selected from the group consisting of nucleic acids specific to species of microorganisms, characteristic of a genetic disorder or disease, HLA types, and sex.

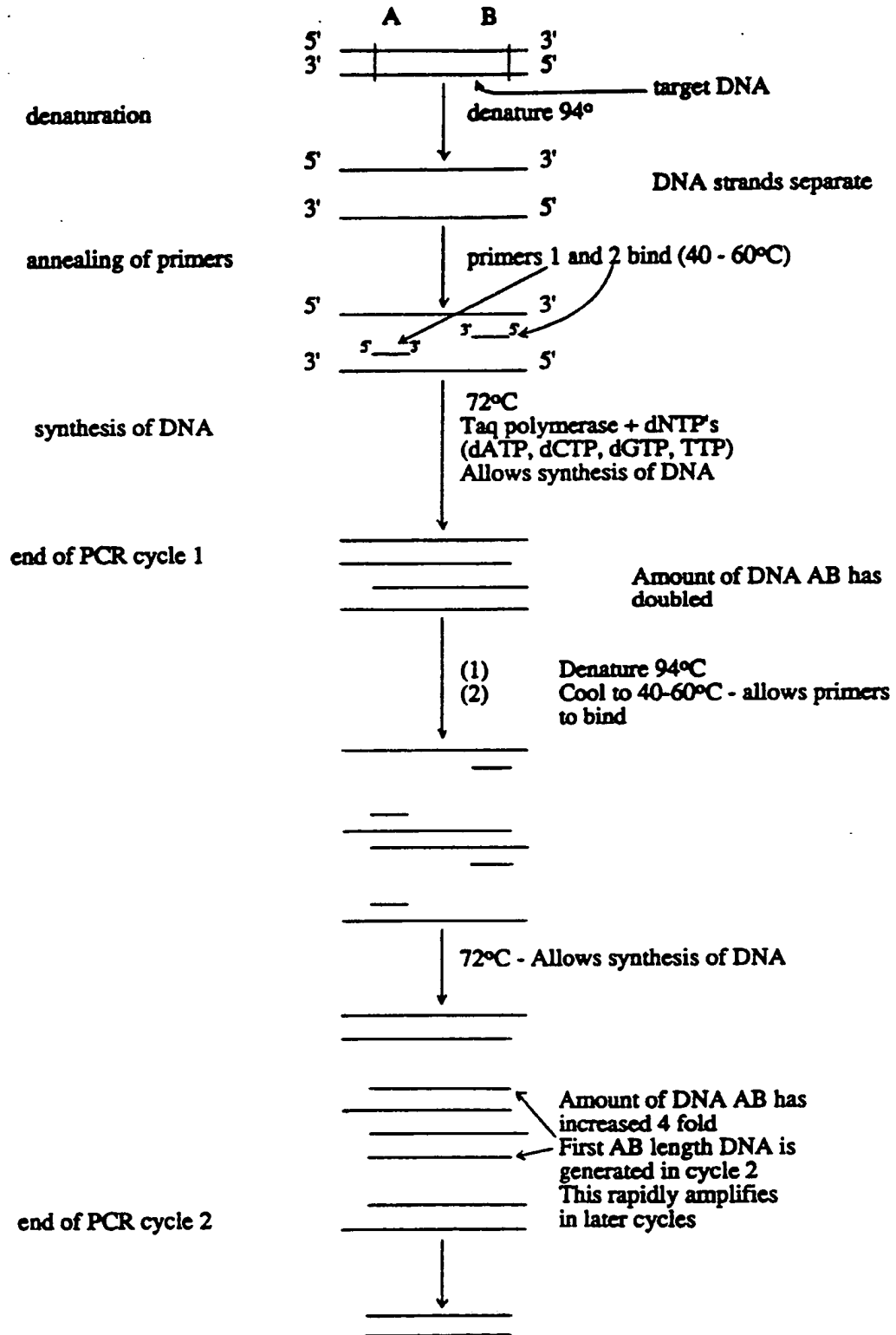
## AMENDED CLAIMS

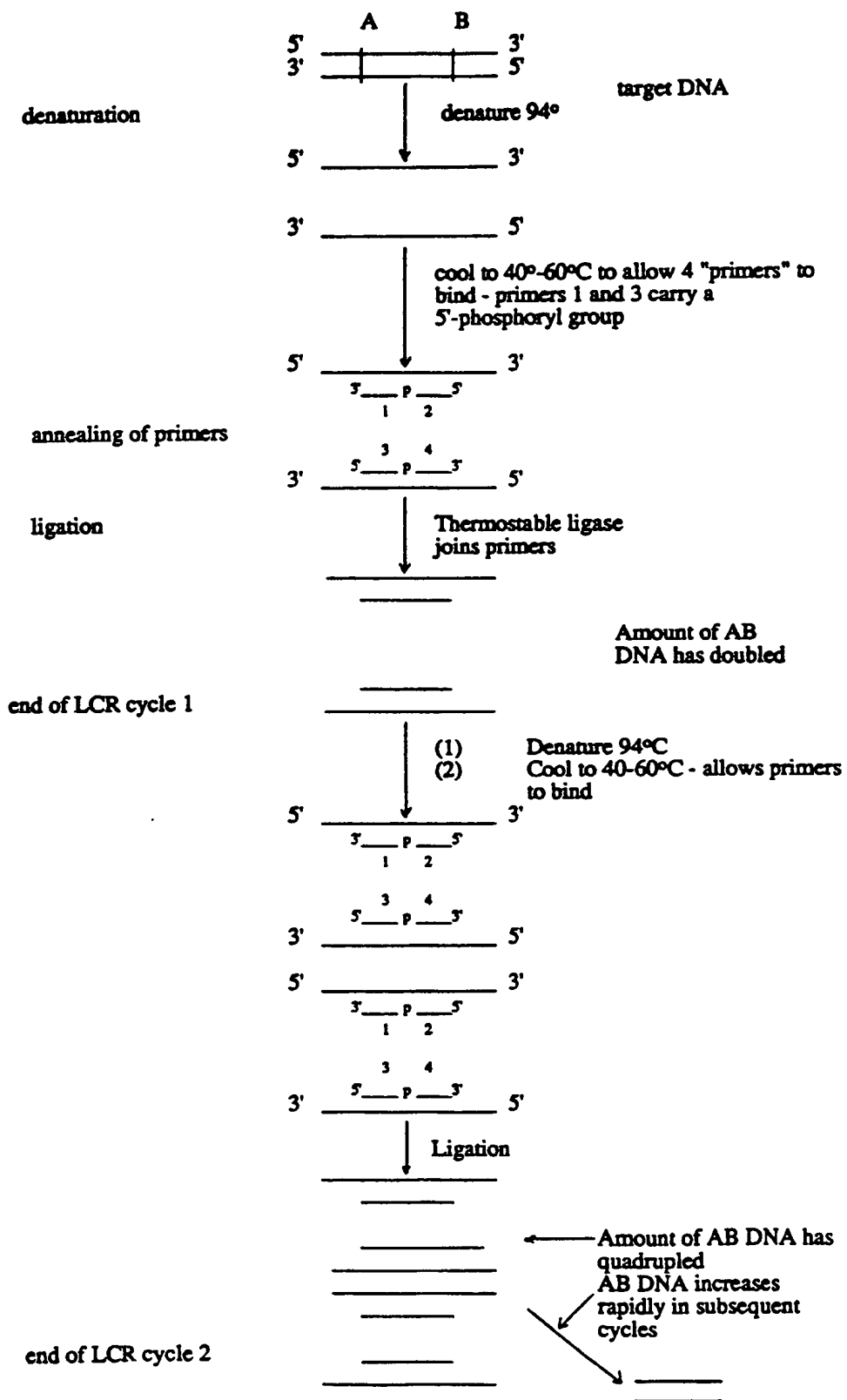
[received by the International Bureau on 27 April 1993 (27.04.93);  
original claims 1,6-17 replaced by new claims 1,6-21 (3 pages)]

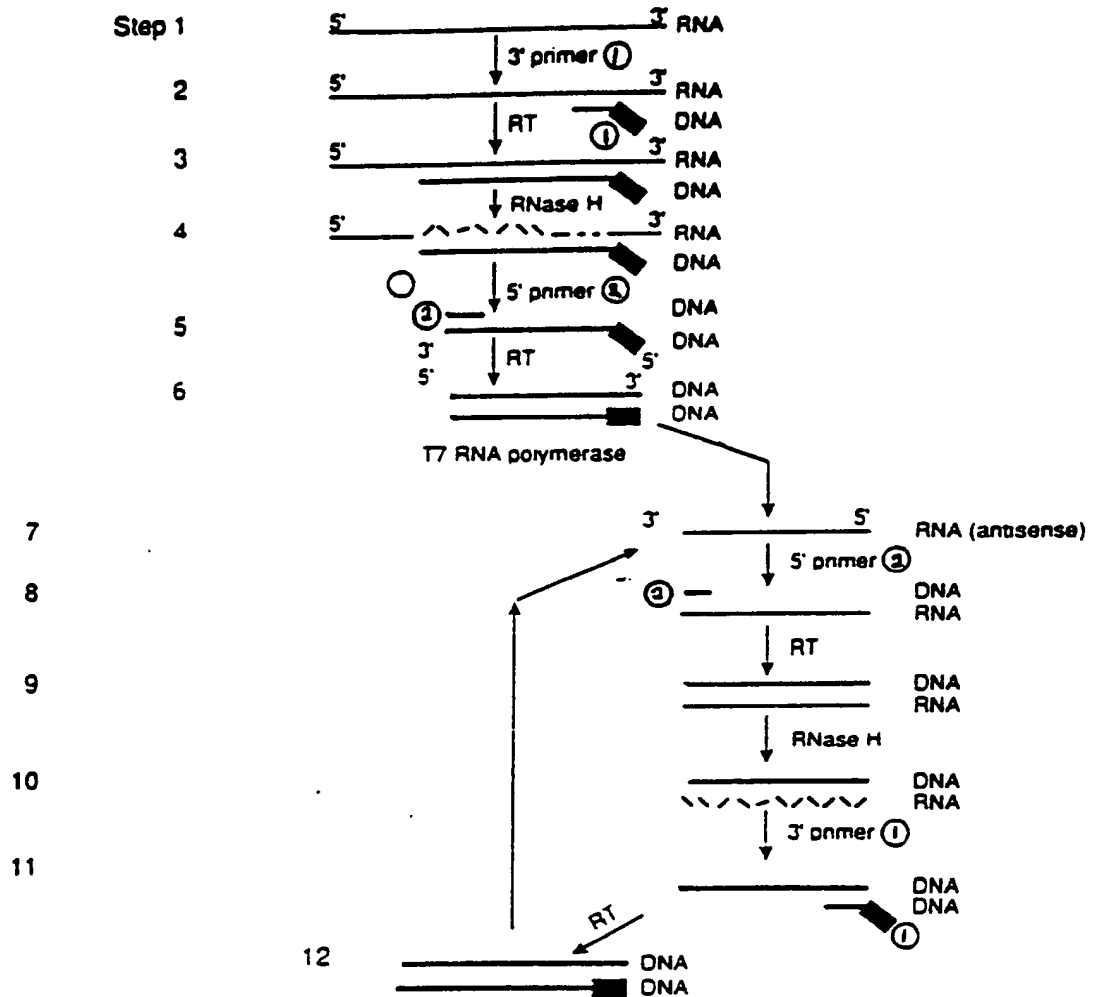
1. A method for detecting a target nucleic acid comprising:  
    providing a first primer hybridizing to the  
    target nucleic acid, wherein the primer is immobilized on a  
    solid phase planar support by a direct chemical linkage  
    between the primer and the solid phase support, wherein the  
    solid phase support forms a part of or is insertable into a  
    container for the sample,  
    providing a second primer hybridizing to the  
    target nucleic acid in the opposite direction, wherein the  
    first primer is labelled with a detectable label,  
    reacting the first and second primers with a  
    sample containing nucleic acids under conditions which allow  
    amplification of the nucleic acids that hybridize to the  
    first and second primers in the container for the sample, and  
    detecting the presence of bound second primer.
2. The method of claim 1 wherein the container is a microtiter  
plate well.
3. The method of claim 1 wherein the first primer is immobilized  
on a solid planar support which is immersible into the  
container.
4. The method of claim 1 wherein the nucleic acid is amplified  
by the polymerase chain reaction.
5. The method of claim 1 wherein the nucleic acid is amplified  
by the nucleic acid sequence based amplification.
6. The method of claim 1 wherein the nucleic acid is amplified  
by the ligase chain reaction based amplification.
7. The method of claim 1 wherein the nucleic acid is amplified  
by the Q $\beta$  replicase based amplification.
8. The method of claim 1 wherein the nucleic acid is amplified  
by the strand displacement amplification.
9. The method of claim 1 wherein the nucleic acid is selected  
from the group consisting of nucleic acids specific to  
species of microorganisms, characteristic of a genetic  
disorder or disease, HLA types, an identity, sex or  
speciation of an organism.
10. The method of claim 1 wherein the label is selected from the  
group consisting of fluorescent labels, biotin, radiolabels,  
enzymes specific for chromogenic substrates and dyes.

11. The method of Claim 1 wherein the label is attached during the amplification process.
12. The method of Claim 1 wherein the label is attached after amplification process.
13. An assay system or kit for detecting a target nucleic acid comprising:
  - a first primer hybridizing to the target nucleic acid, wherein the primer is immobilized on a solid phase planar support by a direct chemical linkage between the primer and the solid phase support,
  - wherein the solid phase support forms a part of or is insertable into a container for the sample,
  - a second primer hybridizing to the target nucleic acid in the opposite direction,
  - wherein the second primer is labelled with a detectable molecule, and
  - reagents for amplification of the sample containing nucleic acids under conditions which allow amplification of the nucleic acids that hybridize to the first and second primers in the container for the sample, and
  - detection of the label on the bound second primer.
14. The assay system or kit of claim 13 wherein the container is a microtiter well plate, further comprising the well plate.
15. The assay system or kit of claim 13 wherein the first primer is immobilized on a solid planar support which is immersible into the container.
16. The assay system or kit of claim 13 wherein the reagents for amplifying the nucleic acid are those for use in the polymerase chain reaction.
17. The assay system or kit of claim 13 wherein the reagents for amplifying the nucleic acid are those for use in the nucleic acid sequence based amplification.
18. The assay system or kit of claim 13 wherein the reagents for amplifying the nucleic acid are those for use in the ligase chain reaction based amplification.
19. The assay system or kit of claim 13 wherein the reagents for amplifying the nucleic acid are those for use in the Q $\beta$  replicase based amplification.

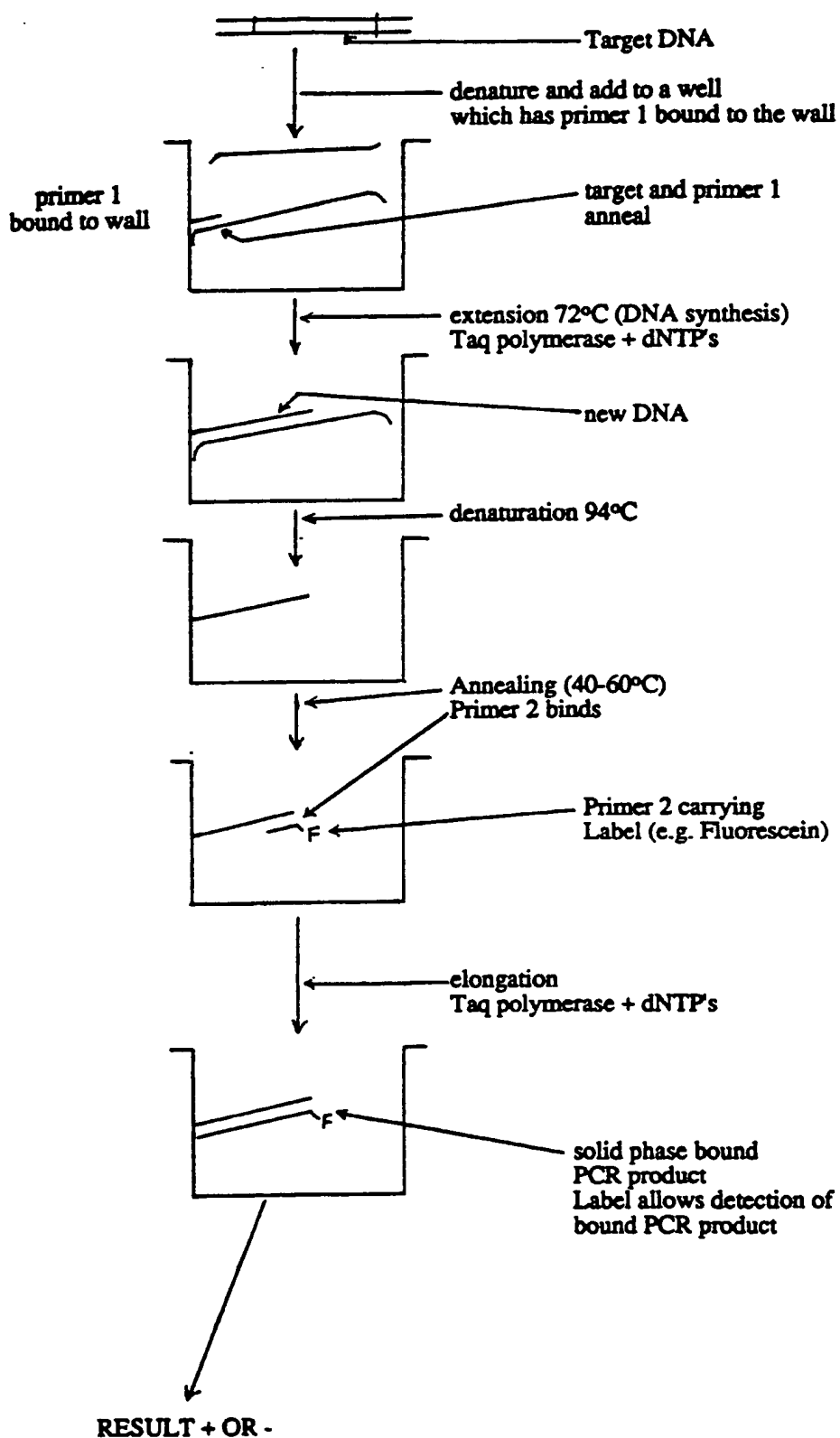
20. The assay system or kit of claim 13 wherein the reagents for amplifying the nucleic acid are those for use in the strand displacement amplification process.
21. The assay system or kit of claim 13 wherein the nucleic acid is selected from the group consisting of nucleic acids specific to species of microorganisms, characteristic of a genetic disorder or disease, HLA types, an identity, sex or speciation of an organism.

**Figure 1 : PCR (Polymerase Chain Reaction) Amplification**

**Figure 2 : LCR (Ligase Chain Reaction) Amplification**

**Figure 3 : NASBA (Nucleic Acid Sequence Based Amplification)**



**Figure 4 : SPA (Solid Phase Amplification)**

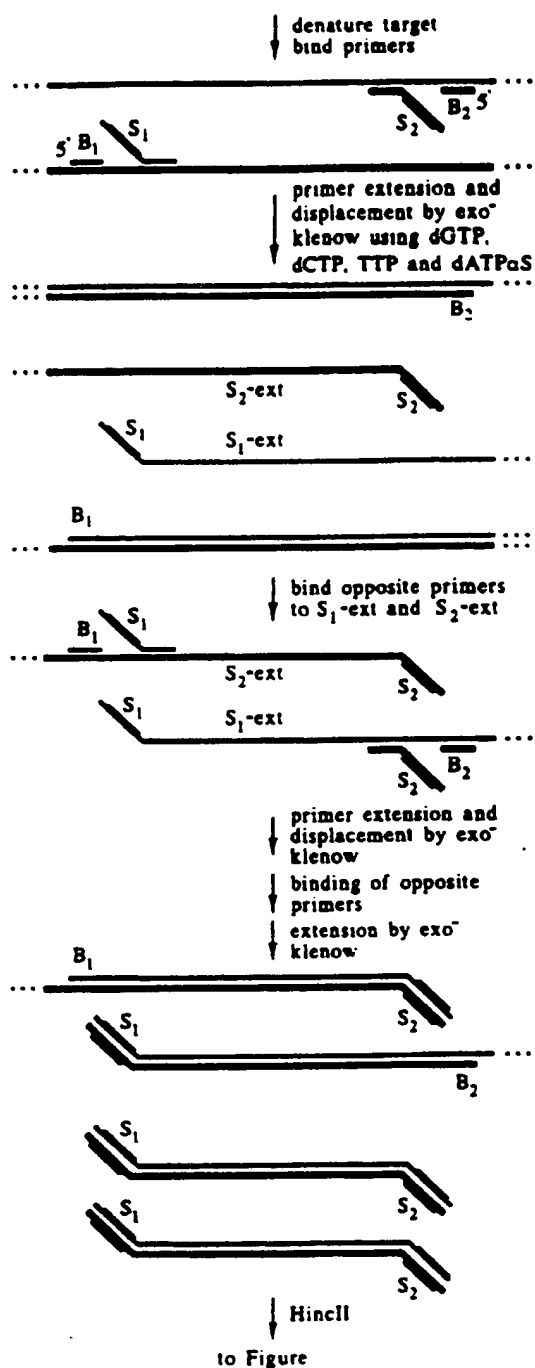
**Figure 5a : SDA (Strand Displacement Amplification)**

Figure 5b : SDA (Strand Displacement Amplification) (cont)

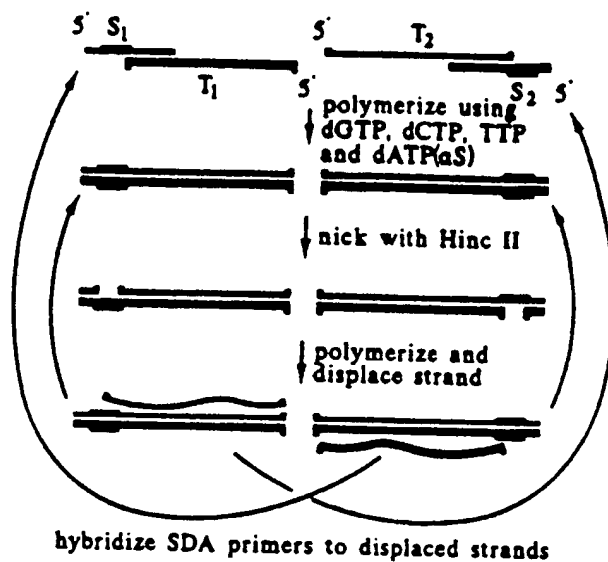
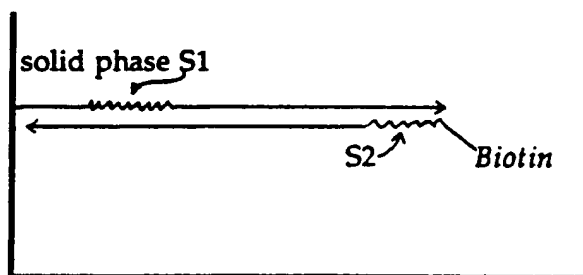


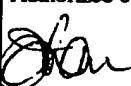
Figure 6 : Final SPA-SDA Product



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU92/00587

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> Int. Cl. <sup>5</sup> C12Q 1/68  According to International Patent Classification (IPC) or to both national classification and IPC					
<b>B. FIELDS SEARCHED</b>  Minimum documentation searched (classification system followed by classification symbols) WPAT, CHEM ABS DATABASES (Keywords as below)  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AU: IPC C12Q 1/68, BIOTECH ABS (Keywords as below)  Electronic data base consulted during the international search (name of data base, and where practicable, search terms used) WPAT, CHEM ABS, BIOTECH ABS, Keywords: Primer, label, immobilized, bound, solid, fixed, amplify/amplification.					
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>					
<b>Category*</b>	<b>Citation of document, with indication, where appropriate, of the relevant passages</b>	<b>Relevant to Claim No.</b>			
X	AU,A,52728/90 (Cemu Bioteknik) 4 October 1990 (04.10.90) See pages 1-3, page 8 and claims.	1-4,8-13,17			
X	The claimed amplification methods are admitted to be known and are equivalent to the PGR method disclosed in the citation.	5-7,14-16			
X	EP,A,370694 (Eastman Kodak Company and Cetus Corporation) 30 May 1990 (30.05.90) See pages 3 and 6.	1-17			
<div style="display: flex; justify-content: space-between;"> <div> <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.         </div> <div> <input checked="" type="checkbox"/> See patent family annex.         </div> </div>					
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%; vertical-align: top;"> <b>* Special categories of cited documents :</b>  <b>"A"</b> document defining the general state of the art which is not considered to be of particular relevance  <b>"E"</b> earlier document but published on or after the international filing date  <b>"L"</b> document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  <b>"O"</b> document referring to an oral disclosure, use, exhibition or other means  <b>"P"</b> document published prior to the international filing date but later than the priority date claimed         </td> <td style="width: 33%; vertical-align: top;"> <b>"T"</b> later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  <b>"X"</b> document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone  <b>"Y"</b> document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art  <b>"&amp;"</b> document member of the same patent family         </td> <td style="width: 33%;"></td> </tr> </table>			<b>* Special categories of cited documents :</b> <b>"A"</b> document defining the general state of the art which is not considered to be of particular relevance <b>"E"</b> earlier document but published on or after the international filing date <b>"L"</b> document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) <b>"O"</b> document referring to an oral disclosure, use, exhibition or other means <b>"P"</b> document published prior to the international filing date but later than the priority date claimed	<b>"T"</b> later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention <b>"X"</b> document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone <b>"Y"</b> document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art <b>"&amp;"</b> document member of the same patent family	
<b>* Special categories of cited documents :</b> <b>"A"</b> document defining the general state of the art which is not considered to be of particular relevance <b>"E"</b> earlier document but published on or after the international filing date <b>"L"</b> document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) <b>"O"</b> document referring to an oral disclosure, use, exhibition or other means <b>"P"</b> document published prior to the international filing date but later than the priority date claimed	<b>"T"</b> later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention <b>"X"</b> document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone <b>"Y"</b> document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art <b>"&amp;"</b> document member of the same patent family				
Date of the actual completion of the international search 5 February 1993 (05.02.93)		Date of mailing of the international search report 16 FEB 1993 (16.02.93)			
Name and mailing address of the ISA/AU  AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA  Facsimile No. (06) 2853593		Authorized officer   J.H. CHAN  Telephone No. (06) 2832340			

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU92/00587

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
Y	AU,A,35622/89 (Paulsen, G. et al) 30 November 1989 (30.11.89) See page 6.	1-17
Y	AU,A,47144/89 (Molecular Diagnostics Inc.) 28 June 1990 (28.06.90) See claim 3.	1-17
Y	AU,A,52695/90 (Gene-Trak Systems) 20 September 1990 (20.09.90) See pages 8 and 9.	1-17
Y	AU,B,46637/89 (633036) (Amrad Corporation Limited) 14 June 1990 (14.06.90) See pages 14 and 15.	1-17
A	AU,A,63290/90 (F. Hoffmann-La Roche AG) 11 April 1991 (11.04.91)	
A	AU,B,53294/86 (599013) (Molecular Diagnostics Inc.) 28 August 1986 (28.08.86)	
A	AU,B,14269/88 (618951) (Arnold L. J. et al) 26 September 1988 (26.09.88)	
A	WO 91/14788 (Chiron Corp) 3 October 1991 (03.10.91)	

**INTERNATIONAL SEARCH REPORT**  
Information on patent family member

International application No.  
**PCT/AU92/00587**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	52728/90	CA	2050681	EP	464067	WO	90/11369
EP	370694	CA	2002076	DK	5821/89	JP	2299600
AU	35622/89	EP	415978	NO	882261	WO	89/11546
AU	47144/89	CA	2004326	DK	6627/89	EP	374665
		JP	3043099				
AU	52695/90	CA	2049043	EP	463036	WO	90/10716
AU	46637/89	CA	2004990	DK	1080/91	EP	447464
		NO	912169	WO	90/06374		
AU	63290/90	BR	9004881	CA	2026280	EP	420260
		JP	3206898	NO	904240	ZA	9007706
AU	53294/86	CA	1272443	DE	3677026	DK	820/86
		EP	192168	ES	552283	ES	8705034
		FI	860744	IL	77926	JP	61195699
		NO	860400	NZ	215214	ZA	8601306
AU	14269/88	GB	2224071	DK	6075/88	EP	281390
		FI	885022	JP	1502319	NO	884847
		PT	86881	WO	88/06633		
WO	91/14788	EP	521111				
END OF ANNEX							

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**